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The Pantothenate-Synthesizing Enzyme, A Metabolic Site in the Herbicidal Action of Chlorinated Aliphatic Acids

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EVIDENCE that β-alanine or calcium pantothenate partially reversed the inhibitory action of several chlorinated aliphatic acids on yeast growth (4) suggested the hypothesis that synthesis of pantothenate is one of the metabolic pathways sensitive to this group of herbicides. The objectives of the experiments reported herein were (a) to determine the mechanism by which the enzymatic synthesis of pantothenate was inhibited by these compounds, (b) to seek evidence for the contributions of this mechanism, *in vivo*, to the herbicidal effects of 2,2–dichloropropionic acid (dalapon), (c) to determine whether pantothenate treatments could overcome the effects of dalapon in plants and (d) to test chloro-substituted analogs of pantoate for herbicidal activity. To achieve the last objective it was necessary to synthesize several new chemicals.

MATERIALS AND METHODS

Preparation and assay of enzyme.

Partially purified preparations of the pantothenate-synthesizing enzyme were obtained from acetone powders of *Escherichia coli*, ATCC 9637, by the procedure described by Novelli (9). Approximately 1 gm of acetone powder provided sufficient enzyme for each experiment. Enzyme activity was measured manometrically at 30° C by determination of acid-liberated CO_2 from pH 8 bicarbonate buffer under a 5-percent CO_2 atmosphere. The manometric technique was feasible because a net production of acid resulted from the overall reaction of synthesis of pantothenate from pantoate, β-alanine and adenosine triphosphate (ATP) (9).

Reagent solutions were adjusted to pH 8 with KOH prior to addition to Warburg vessels. The K pantoate solution was prepared from *l*-pantoyl lactone by the procedure of Maas (8). All solutions except ATP were mixed in the main compartment of the vessel

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during the equilibration period; ATP was added from a side arm to initiate the reaction. For routine analysis the composition of the final 3.0-ml reaction volume was 0.1 M KCl, 0.01 M $MgSO_4$, 0.01 M ATP, 0.02 M β-alanine, 0.02 M K pantoate, 0.066 M $KHCO_3$ and enzyme.

Herbicidal chemicals[3] used in enzyme studies were obtained as purified acids and solutions containing them were adjusted to pH 8 with KOH. The chemicals with their abbreviated designations and purities were as follows: 2,2–dichloropropionic acid (dalapon), 99+ %; 2,3–dichloroisobutyric acid (DCIB), 99+ %; trichloroacetic acid (TCA), USP; 2,2,3–trichloropropionic acid (2,2,3–TPA), 99+ %; and 2,3,3′–trichloroisobutyric acid (TCIB), 90% [impurities of 2,3,3–trichloroisobutyric and 2,3–dichloroisobutyric acids].

Liberation of CO_2 was proportional to enzyme concentrations up to rates in excess of 300 µl CO_2/hr. Greatest accuracy was obtained with enzyme concentrations which liberated about 250 µl CO_2/hr, and this quantity of enzyme was selected for subsequent investigations. ATPase, which occurred as a contaminate in all enzyme preparations, liberated an additional 20 to 60 µl CO_2/hr. Appropriate corrections were made for ATPase in all rate determinations. The ATPase activity was not affected by any of the chemicals used to inhibit the pantothenate-synthesizing enzyme. Enzyme-dissociation constants for substrate and inhibitory chemicals were determined for each of the individual enzyme preparations by the Lineweaver and Burk method of analysis (7) from the combined data of three independent experiments.

Seedling growth tests.

Barley (*Hordeum vulgare* var. Dayton, C. I. 9517) and oats (*Avena sativa* var. Clinton 59) were germinated and grown under greenhouse conditions in 8-oz polyethylene cups containing perlite saturated with Hoagland's solution. Moisture levels were maintained by surface applications of tap water. Barley seedlings were treated at the two-leaf stage, 9 days after planting. The tip of the first leaf was removed and the cut end of the leaf immersed in a vial containing 10 ml of a 0.02 M metabolite solution. Vials were removed 8 days later. The day following initiation of metabolite treatments, a sodium salt solution of dalapon was applied to the perlite surface of each cup in amounts equivalent to 4 lb/A, active ingredient (1.73 mg/cup). Plants were dissected 5 weeks after planting to determine the number of developed tillers that contained chlorophyll and were 1 cm or more in length.

Oat seeds (4 seeds/cup) were planted on a saturated perlite surface. The seeds were sprinkled uniformly with dry Ca pantothenate (0.1 gm/cup) and then covered with an additional 0.5 cm of perlite. Sodium dalapon was applied to the perlite surface in 10 ml of solu-

[3]The experimental compounds used in these studies were supplied by research personnel of the following organizations: Amchem Products, Inc., Ambler, Pa.; American Cyanamid Co., Stamford, Conn.; Dow Chemical Co., Midland, Michigan; Rohm and Haas Co., Philadelphia, Pa.

tion on the following day. Total fresh weight of tops from each cup was determined 3 weeks after planting.

Ryegrass germination tests.

Seeds of Italian ryegrass *(Lolium multiflorum)* were germinated in Petri dishes at 20° C. Fifty seeds were planted in each dish on three 9-cm disks of Whatman No. 2 filter paper, soaked for 1 hour in 10-ml solutions of chemicals. The molar concentrations reported for chemicals refer to concentrations of the 10-ml solutions. Excess solution was poured off at time of planting. Approximately 24 and 48 hours after planting all dishes were exposed to white fluorescent light for 15 min. This procedure resulted in more than 95 percent germination of the seeds. Seedling development was arrested by freezing after a 96-hour growth period. The lengths of the coleoptile and the root of each seedling were determined immediately after thawing of individual dishes.

Syntheses of new metabolic analogues.

2–chloro–4–hydroxy–3,3–dimethylbutyric acid, γ–lactone, *dl*–, (Table 4, II). The method was adapted from that of Darzens (1) with dependence on explanations by Gerrard (3). Into a 1-L reaction flask were put 100 gm (1.00 molar equivalent, *ME*) of *dl*–pantoyl lactone, 60 ml of (0.97 *ME*) pyridine, and 300 ml of anhydrous Et_2O. While this mixture was magnetically stirred and kept below 10° C with an ice bath, 96 gm (1.05 *ME*) of thionyl chloride dissolved in 100 ml of Et_2O was dropped in gradually over 1 hr, causing pyridine hydrochloride precipitation. The mixture stood overnight at room temperature with a ball-joint stopper acting to release pressure and prevent entrance of humid air. The Et_2O was distilled; then the warm system became a single-phase liquid that included the pyridine hydrochloride. The mixture was stirred at 130° for 15 min to disengage SO_2 from an intermediate considered to be *dl*-pantoyl lactone 2–0–chlorosulfinic ester. The transformation was completed during further stripping of volatiles with vacuum at this temperature, and by distillation at 122–140°/25 mm in a special still. To prevent obstruction of the still by keeping the condensate sufficiently melted, the distilled vapors passed directly to a receiver and then upward to a reflux condenser so that the receiver trapped reflux return at the condensation temperature. The distilled mixed solids, consisting of pyridine hydrochloride and product, were partitioned with water and benzene in a separatory funnel. The benzene layer was washed with water, dilute HCl, water, dilute equimolar proportions of $NaHCO_3$ and Na_2CO_3, and water; shaken with diatomaceous filter aid, anhydrous Na_2SO_4, and carbon; filtered; the solvent volatilized; and the product redistilled in the same still at 120–121°/14 mm. The yield was 84.5 gm, 74% of theory, of a colorless amorphous wax melting at 71.5° and having an aromatic odor of moderate intensity. Theory for $C_6H_9O_2Cl$, M. W. 148.60, Cl 23.86%, C 48.50%, H 6.11%; found Cl 23.77%, C 48.58%, H 5.74%. By infrared spectrum of the clear wax, pressed warm and solidified, the carbonyl

band center was at 1788 cm $^{-1}$ with maximum at 1790 (illustrating an α-chlorine shift from a corresponding 1775 center for *dl*-pantoyl lactone, pressed clear). Identifying multiplet maxima were at 1468, 1364, 1260, 1140, 1008, and 930; and a series of sharper peaks were at 901, 874, 844, 829, 798, 791, 692. and 660.

N–(2–chloro–4–hydroxy–3,3–dimethylbutyryl)–β–alanine, *dl*-, calcium salt (of acid IV, Table 4). The method was similiar to that published for calcium *D*(+)–pantothenate (12). With dry methanol to a volume of 50 ml, 1.03 gm (0.90 *ME*) of sodium was first dissolved, then 5.35 gm (1.2 *ME*) of β–alanine was partly dissolved by refluxing, and after cooling to room temperature, 7.43 gm (1.0 *ME*) of the lactone II was added. The mixture was machine-shaken, left 3 days at room temperature and filtered. The methanol was removed with vacuum below 30° C. The residue was shaken 1 hour with 2.7 gm (1.2 combining equivalents, *CE*) of anhydrous oxalic acid dissolved in 35 ml of absolute ethanol and filtered with diatomaceous filter aid. The filtrate was diluted with an equal volume of water, 6.6 gm (2.6 *CE*) of precipitated fine calcium carbonate added, shaken 1 hour with a pressure-releasing stack attached, and filtered. The filtrate was concentrated below 40° with vacuum, and the water more completely removed azeotropically by repeated similar concentrations after adding portions of 1:1 benzene and absolute ethanol. The concentrate was dissolved in a minimum of methanol, acetone was added until near precipitation, and the mixture was poured into acetone, giving a gummy precipitate. A specimen was manipulated between the surface of a ground-glass joint, allowing moderate heatless orientating seizures until hardening was evident; then scrapings of this were stirred into the main mass as crystalization seed. After crystalization had proceeded overnight, the lumps were crushed to a white powder, washed with acetone decantations, gathered on a filter, and air-dried; yielding 8.97 gm, 70% of theory, of a white powder that no longer seemed either hygroscopic or cohesive. Theory for $C_{18}H_{30}O_8N_2Cl_2Ca$, M. W. 513.45, Cl 13.81%, N 5.46%, Ca 7.81%. Found on a desiccated sample, M. P. 146–147°, Cl 13.14%, N 5.38%, Ca 7.55%. Mull spectra gave OH and/or NH bands at 3292 and 3094 cm $^{-1}$, and others at 1461–1414, 1370 and 1330, in hexachlorobutadiene; in Nujol the primary amide maxima were at 1661 and 1595, the ionized carbonyl maximum at 1550, and others at 1233, 1057, 1019, 988, 946, 914, 889, 815, 775, 682, and 657. For comparison Nujol mull bands of calcium *D*-pantothenate were found at 1653 (center of a 1659- and 1647-inflected amide-I type envelope) and 1560 (center of a broad band enveloping the amide-II and ionized carbonyl bands).

2–Chloro–4–hydroxy–3,3–dimethylbutyric acid, *dl*- (Table 4, VI). The lactone II was hydrolyzed in a Waring Mixer with conditions selected to make completion of hydrolysis evident by disappearance of turbidity; and special precautions were taken to diminish dehalogenation by alkali and to diminish re-lactonization by catalysts, heat, and dehydration. Near the final step an exposure to benzene was limited to a few minutes, because failure of a previous attempt was

attributed to re-lactonization within a few hours by benzene or associated catalysts. The final pure crystals were stored at low temperature and hastily used; the need for this was confirmed when the rate of reversion to the lactone was later determined and found expressable by a half-life of 43 days at 25° C.

The speed of a Waring Mixer was reduced with a variable transformer and its detachable chamber preheated in an oven to 100° C. In this was blended 300 ml of boiling water, 2 drops of a non-ionic surfactant (Triton X–100), 30 ml of ethanol, and 38.0 gm (1.00 *ME*) of the lactone II. After starting emulsification above the lactone M.P., ice was added until the temperature stabilized at 25°; and 20 gm of NaCl was added. Aqueous NaOH, 92.7 ml of 3.89 *N* (1.41 *ME*), was added and the blending continued for a total of 30 min or twice the time required for the solution to become nearly clear, as seen beneath the foam with the motor stopped. The excess alkali was then inactivated with 58.1 ml of 2.024 *N* (0.46 *ME*) HCl, theoretically leaving the product 5% acid-form and 95% neutralized. Diatomaceous filter aid and carbon were blended in and the solution filtered to give a clear neutral solution. To this in a separatory funnel were added 200 ml of Et_2O and 114 ml of 2.024 *N* (0.90 *ME*) HCl, the product account becoming 90% acid-form, 5% neutralized, and 5% removed. Three Et_2O extractions were made; each was washed with fresh saturated NaCl solution in a second funnel, and the used rinses were returned to the first funnel. The combined ether extracts were treated with anhydrous Na_2SO_4, filtered, and concentrated on a steam bath, without allowing the last of the ether to evaporate as a protection against heat exceeding 40°. Benzene, 20 ml, was added and the concentration continued with a 40° bath and vacuum to give a thick slurry of crystals. With haste to limit the benzene exposure the crystals were transferred to a filter and washed with four 20-ml portions of benzene, followed by five of petroleum ether, and then dried at 42°/50 mm for 30 min. The yield was 29 gm, 68% of theory, of odorless white crystals. Theory for $C_6H_{11}O_3Cl$, M. W. 166.61, and Cl 21.28%. Found Cl 21.28%, alkalimetric titration for the free acid 99.4%, ash below 0.04%, M.P. 90–91° (lactonizing influences probably could vary the M.P.) Nujol mull maxima were at 3400 (ROH); 2668, 2574, and 1709 (RCOOH); 1307, 1287, 1234, 1199, 1185, 1137, 1043, 980, 959, 923, 914, 825, 786, 720, 712, and 665 cm^{-1}. The band of lactone II at 1788, found a sensitive indication of reversion, only slightly modified the curvature.

After a specimen of the acid, VI, had reverted toward its γ–lactone, II, for 112 days at room temperature, and additionally for 30 min in a 90° C oven, for drying and melting to get spectrum of the clear wax, the infrared bands were found identical throughout the salt region with those of pure lactone II. This substantiated the intactness of the α–Cl in VI, which was not simply ascertained by a carbonyl frequency shift as for II, because in VI the γ–OH and COOH interactions exerted an influence opposite and approximately balancing that of α–Cl.

4–Chloro–2–hydroxy–3,3–dimethylbutyric acid, ethyl ester, *dl*-

(Table 4, I). The transformation of a γ-lactone to a γ–chloro ester was modeled after an example by Levene and Mori (6); they did not report the yield, and that here was small. With a solution of 13 gm (0.10 *ME*) of *dl*–pantoyl lactone in 46 gm of dry ethanol, saturation with HCl was approached by introducing 35 gm of dry gas. After standing 5 days at room temperature, stoppered with a ball joint as a safety precaution, it was poured into ice water and quickly extracted with Et_2O. That extraction, having scavenging stages, was not sufficiently discriminative; infrared indicated the residue was about 1:1 product and pantoyl lactone. The residue was redissolved in petroleum ether, washed with 4 water extractions, and distilled, giving at 86°/2 mm 1.8 gm, 9% of theory, of a colorless liquid. Theory for $C_8H_{15}O_3Cl$, M. W. 194.67, Cl 18.21%. Found, Cl 17.61%. Six foreign infrared bands corresponding to *dl*–pantoyl lactone diminished to insignificance during purification; therefore, from the chlorine content the product was concluded to be 96.7% pure, the remainder being *dl*–pantoyl lactone, which was tolerable for the purpose served. Characterizing maxima of the undiluted liquid were at 3500 (OH), 1729 (CO), 1472, 1451, 1393, 1372, 1273, 1217, 1119, 1084, 1018, 906, 863, 806, and 737 cm^{-1}.

Results

Enzyme studies.

Inhibition of the enzymatic synthesis of pantothenate by dalapon was determined in combination with β–alanine by varying the substrate concentrations from 0.00167 M to 0.02 M. When these data were plotted graphically according to the method of Lineweaver and Burk (7) a family of parallel lines was obtained (Figure 1, upper). This type of inhibition, known as uncompetitive inhibition, indicated that the inhibitor did not couple with the enzyme at the point of β–alanine attachment. Data from a similar experiment testing for interaction between pantoate and dalapon gave a typical competitive inhibition test (Figure 1, lower). These data show that dalapon inhibited the enzymatic synthesis of pantothenate by competing wtih pantoate for a site on the enzyme.

A comparison of the enzyme-dissociation constants obtained with the individual enzyme preparations shows that dalapon and the natural substrate, pantoate, have approximately the same affinity for the enzyme. For three independent preparations of the enzyme the dalapon-enzyme dissociation constants (K_i) were 0.0021, 0.0025, and 0.0106 M. The corresponding dissociation constants (K_m) for pantoate were 0.0032, 0.0041, and 0.0060 M respectively. With a fourth preparation the K_m for pantoate was 0.0025. This variability in K_m among preparations is within the range reported previously (9).

Each of the five chlorinated aliphatic acid herbicides inhibited the pantothenate-synthesizing enzyme by competing with pantoate (Figure 2). The enzyme dissociation constants for the five herbicides were determined. In order of increasing affinity for the enzyme, the herbicides and K_i values were dalapon 0.0021 M, DCIB 0.0019 M,

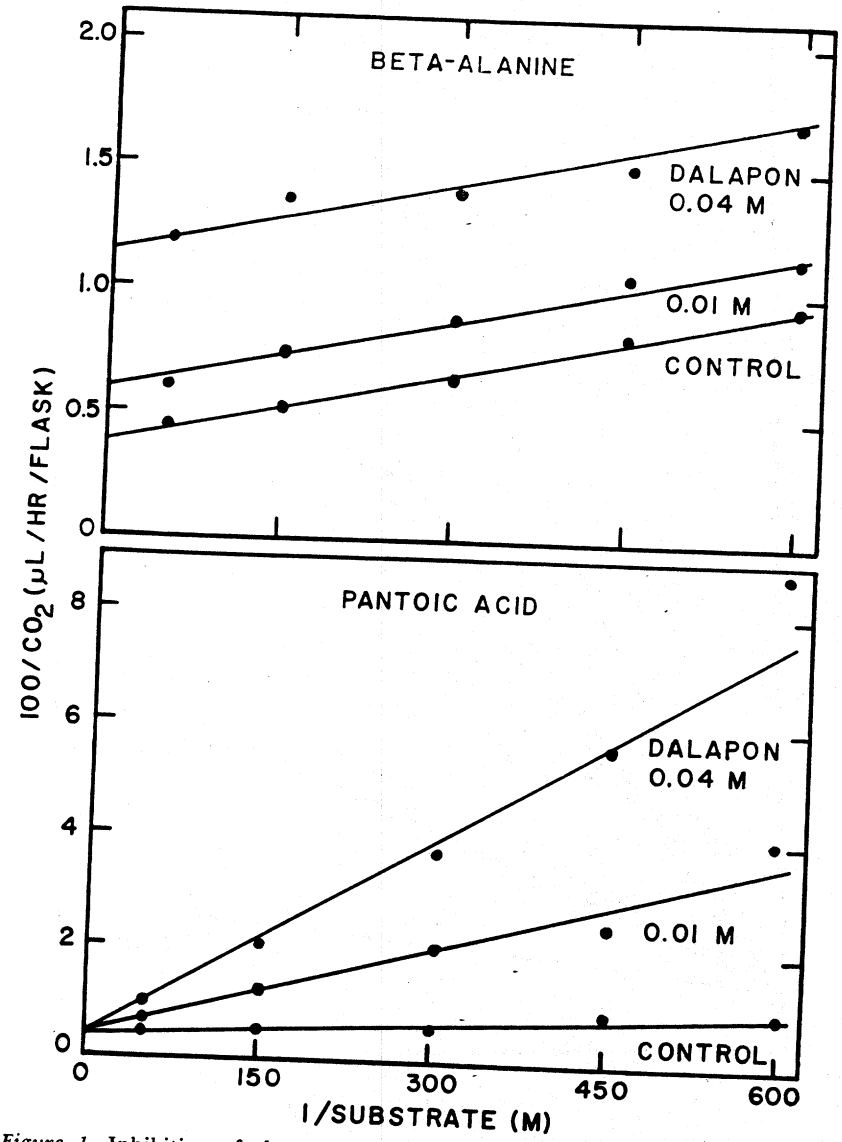

Figure 1. Inhibition of the enzymatic synthesis of pantothenate by dalapon shows the inhibitor to be uncompetitive with β-alanine and competitive with pantoate for a site on the enzyme.

2,2,3–TPA 0.0006 M, TCA 0.0006 M and TCIB 0.0003 M. The lowest constant represents the greatest toxicity. Therefore, the trichloro-compounds were obviously stronger inhibitors than the corresponding dichloro-compounds. The dissociation constant for pantoate

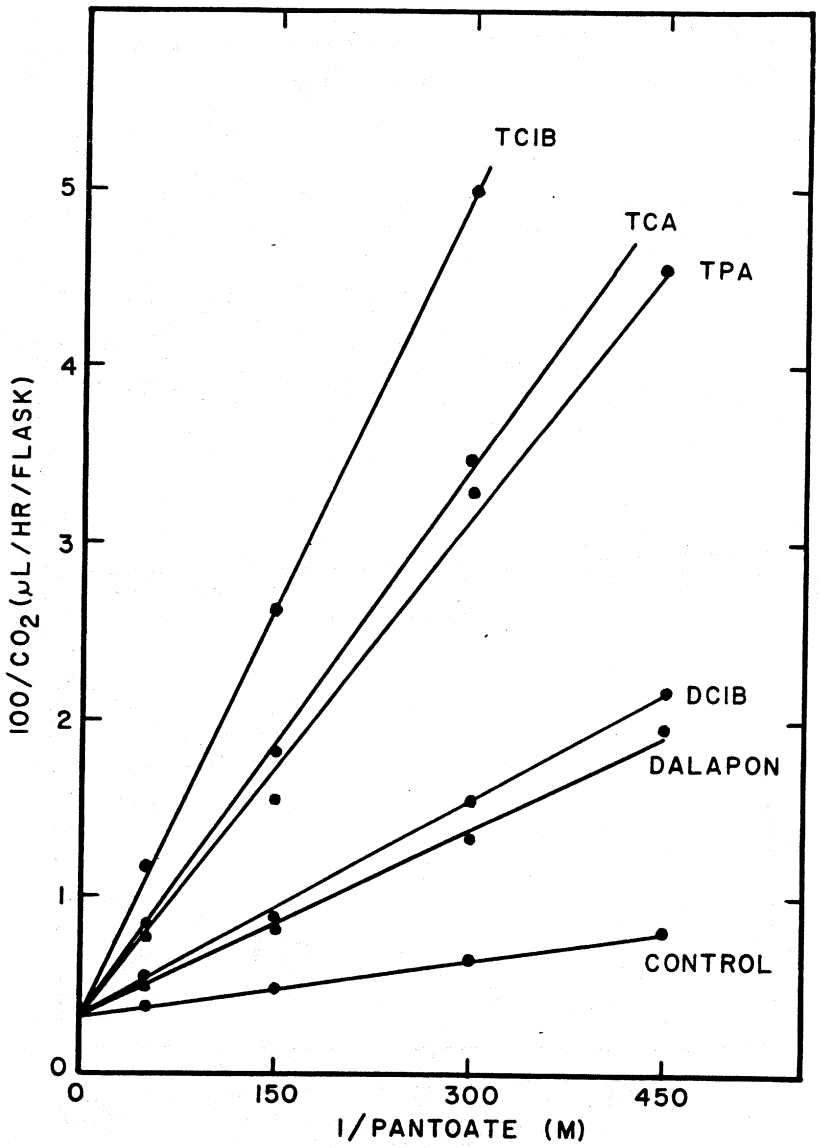

Figure 2. Competitive inhibition test for pantoate and five herbicides. Inhibitor concentrations were 0.005 M for 2,3,3'-trichloroisobutyrate (TCIB); TCA; 2,2,3-TPA; 2,3-dichloroisobutyrate (DCIB); and dalapon.

(K_m) on this preparation was 0.0032 M. Each of the herbicides had an affinity for the enzyme greater than the affinity of the natural substrate.

DL-2-chloro-4-hydroxy-3,3-dimethylbutyrate is an analog of

pantoate in which the α–hydroxyl group of the metabolite was replaced with chlorine. This compound did not replace pantoate as a substrate for the reaction and it inhibited pantothenate synthesis by competition with pantoate. The enzyme preparation used for the data presented in Figure 3 had a K_m for pantoate of 0.0060 M.

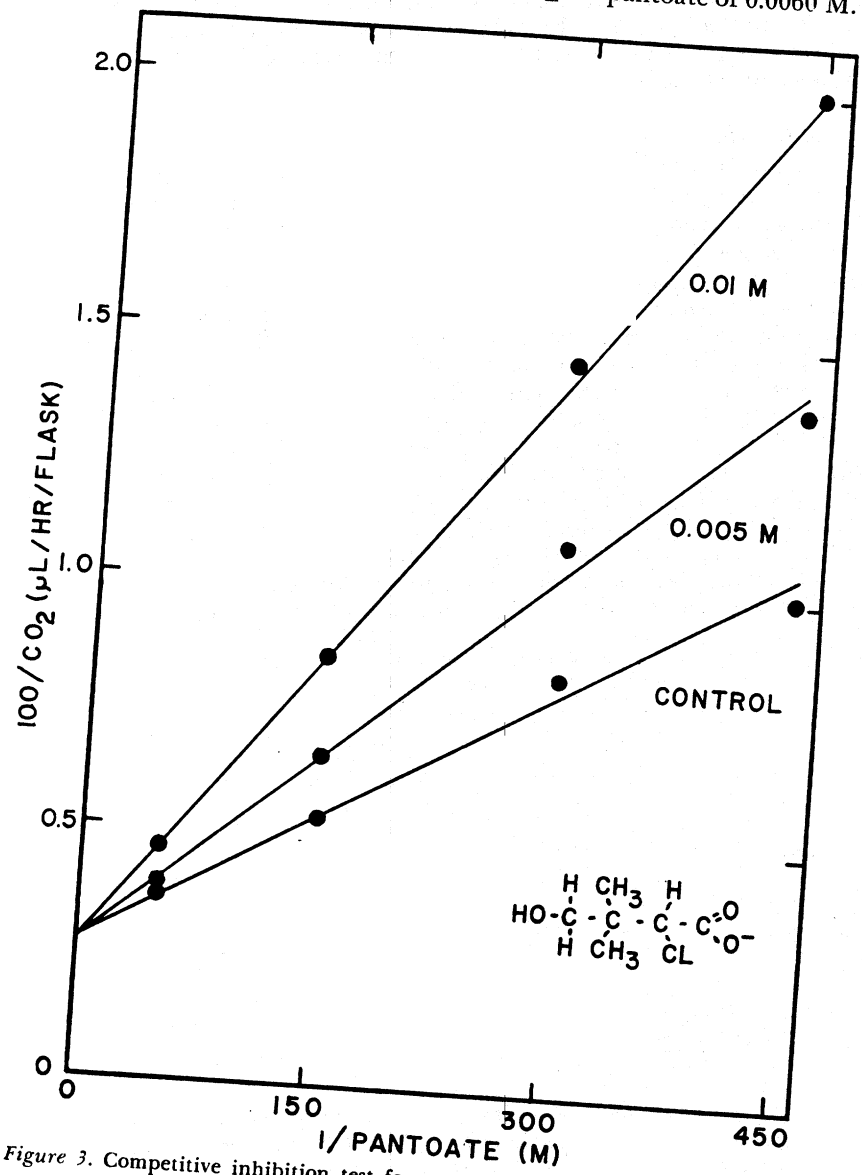

Figure 3. Competitive inhibition test for pantoate and the α-chloro analog of pantoate (structure shown).

On the same preparation the K_i values for dalapon and DCIB were 0.0106 and 0.0064 M, respectively; the K_i values for the pantoate analog calculated from the 0.005 and 0.01 M plots in Figure 3 were 0.0069 and 0.0084 M, respectively. Limited data obtained with a more sensitive enzyme preparation showed a K_i value for the analog slightly less than the K_m value. Therefore, the inhibitor obtained by substituting chlorine for the α–hydroxyl of *dl*–pantoate appears to have about the same affinity for the enzyme as does pantoate. Furthermore, its activity as an enzyme inhibitor is comparable with that of two important herbicides.

Studies with intact plants.

To examine the hypothesis that dalapon inhibits pantothenate synthesis *in vivo,* experiments were designed to determine whether any of the three metabolites could antagonize the effects of dalapon on growth of plants.

Barley. Both pantoate and pantothenate caused significant reduction in the abnormal tiller development on barley plants which resulted from application of sublethal concentrations of the sodium salt of dalapon (4 lb acid equivalent/acre). Untreated plants grown in the greenhouse during the winter developed an average of two tillers per plant in a 5-week growth period. Leaf treatment with water, β–alanine, K pantoate or Ca pantothenate did not alter the number of tillers developed; but plants grown in cups to which dalapon was applied to the perlite surface when seedlings were 10 days old had an average of eight developed tillers per plant 25 days after treatment. There was no evidence that β–alanine could antagonize the dalapon effect on tiller development; however, both pantoate and pantothenate reduced tiller counts significantly (Table 1).

Table 1. Antagonism between metabolites and dalapon as expressed by tiller development in barley.

Leaf treatment[a]	Dalapon[b] lb/A	Tillers per plant[c]
None	0	2.0
Water	0	2.1
β-alanine	0	1.8
Potassium pantoate	0	2.0
Calcium pantothenate	0	2.1
Water	4	7.8
β-alanine	4	8.0
Potassium pantoate	4	5.3
Calcium pantothenate	4	3.2
	LSD 5% level	1.86
	1% level	2.48

[a]Clipped leaf of 9-day-old seedlings immersed for 8 days in 10 ml of 0.02 M solution of metabolite.
[b]Applied to perlite surface of cups containing 10-day-old seedling.
[c]Average of a total of nine 5-week-old plants from two independent experiments with three and six replications.

The ability of Ca pantothenate to antagonize dalapon was observed in all instances. In other experiments, 0.01 M $CaCl_2$ could not replace Ca pantothenate as an antagonist in this test.

The tiller response in plants receiving the dalapon-pantoate combination of treatments was variable. The variation in response may have been due to insufficient uptake and translocation of pantoate in some plants. In all instances pantoate caused some damage to the leaf receiving the metabolite. The treated leaves developed chlorotic areas some distance above the surface of the metabolite solution and desiccation of leaf tissue often followed. In many plants the desiccated areas enlarged sufficiently to prevent further uptake of the metabolite. Pantoate damage to the treated leaf is apparent in Figure 4. This figure also shows the general appearance of plants receiving the dalapon-metabolite treatments as compared with that of an untreated plant.

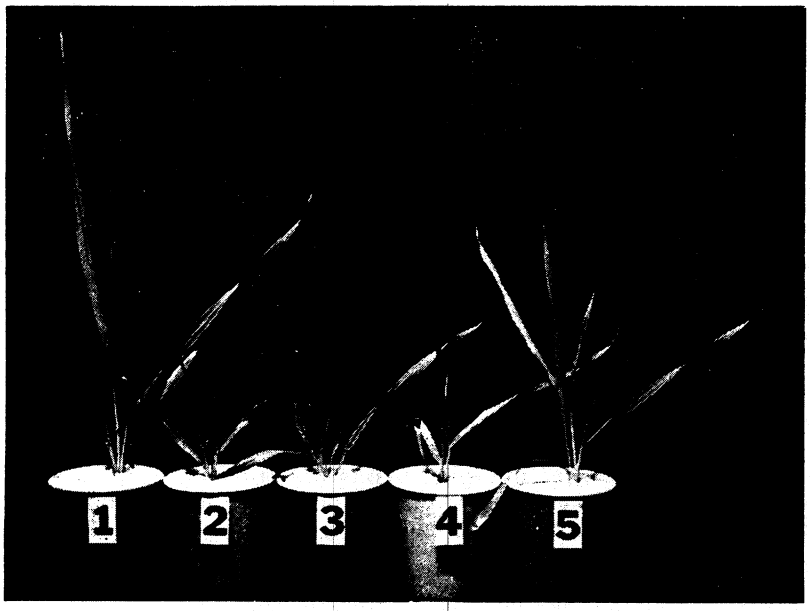

Figure 4. General appearance of 5-week-old barley plants treated with dalapon (4 lb/A applied to perlite surface 10 days after planting) and metabolites (cut leaf of 9-day-old seedlings immersed in 0.02 M solutions for 8 days). (1) Untreated control. (2) Dalapon plus water. (3) Dalapon plus β-alanine. (4) Dalapon plus K pantoate. (5) Dalapon plus Ca pantothenate.

None of the metabolites completely overcame dalapon inhibition of growth (Figure 4). Pantothenate had obviously altered the direction of growth of dalapon-treated plants, but fresh-weight measurements indicated little or no effectiveness in overcoming dalapon inhibition of total growth.

Oats. Ca pantothenate powder was placed over the oat seeds (4 seeds/cup) at the time of planting and the fresh weight of tops was determined 3 weeks later. The data in Table 2 represent the average fresh weight of tops per cup. Both winter and summer growing

conditions are represented in the growth periods of the three individual experiments. Analysis of variance, performed on the combined data, showed the interactions between Na dalapon and Ca pantothenate to be highly significant. Pantothenate alone did not affect growth of the young oat seedlings in the absence of dalapon; but pantothenate did partially offset the inhibitory effects of dalapon on growth of tops (Table 2). Considerable variation was observed

Table 2. Antagonism between calcium pantothenate and dalapon on growth of oats.

Dalapon[b] lb/A	Fresh weight of tops, gm[a]	
	Without pantothenate	With pantothenate[c]
0	2.7	2.6
2	1.74	2.48**
4	0.97	1.58**
LSD 5% level		0.330
1% level		0.437

[a]Average weight per cup. Each cup contained four 3-week-old plants. The data include three separate experiments with six replications each.
[b]Applied 24 hours after planting.
[c]0.1 gm/cup applied to seeds at time of planting.

among replications within individual experiments. This variability probably results from physical or chemical processes other than pantothenate metabolism which are involved in the plant response even at low dalapon concentrations. At higher dalapon concentrations (8 lb/A) very little growth was obtained even in plants receiving pantothenate. This illustrates further the inability of pantothenate to offset completely the toxic action of dalapon.

Ryegrass. The only evidence obtained for antagonism between dalapon and β–alanine in higher plants was observed in the growth of ryegrass seedlings. β–alanine at high concentrations was inhibitory to growth of both roots and coleoptiles (Table 3). Dalapon also

Table 3. Antagonism between β–alanine and dalapon as expressed by growth of ryegrass seedlings.

β-alanine concentration	Coleoptile growth, mm Dalapon conc.		Root growth, mm Dalapon conc.	
	0	0.001M	0	0.001 M
0	11.6	4.7	26.2	13.5
10^{-3}M	9.5	5.3	18.8	13.5
10^{-2}M	8.1	5.7	8.7	7.5

LSD's, 5% level of probability, based on data from 4x4 factorial experiment, 50 seeds per treatment: coleoptiles, 0.8 mm; roots 1.1 mm.

inhibited growth of both organs. However, when seeds were exposed to a combination of the two chemicals, the inhibitions were not additive and growth of coleoptiles was observed to be slightly greater than in dalapon alone. If the dalapon-inhibitory actions shown in Table 3 are expressed as percentage inhibitions of the corresponding

growth levels obtained with β–alanine alone, the inhibition caused by 0.001 M dalapon on coleoptiles was reduced from 60 percent to 44 percent to 30 percent and on roots from 49 percent to 28 percent to 12 percent at β–alanine concentrations of 0, 0.001, and 0.01 M, respectively. Such evidence for antagonism between the two chemicals suggests that both were affecting a single metabolic pathway. These effects might be expected if one assumes that β–alanine protects some sensitive growth site(s) against the inhibition by dalapon despite the fact that β–alanine is inhibitory.

Metabolic analogs.

The ryegrass germination-growth test was employed for a preliminary evaluation of the herbicidal activity of several pantoate and pantothenate analogs listed in Table 4. Four of the chemicals are new

Table 4. Toxicity of dalapon and metabolic analogs to root growth of ryegrass seedlings.

Number	Structure of chemical	Percent inhibition at conc. of	
		0.005 M	0.05 M
I	$Cl \cdot CH_2 \cdot \underset{\underset{CH_3\ OH}{|}}{\overset{\overset{CH_3}{|}}{C}} \cdot CH^a \cdot CO \cdot O \cdot CH_2 \cdot CH_3$	97	100
II	$CH_2 \cdot \underset{\underset{CH_2\ Cl}{|}}{\overset{\overset{CH_3}{|}}{C}} \cdot CH^a \cdot CO$ (O bridge)	77	100
III	$CH_3 \cdot \underset{\underset{Cl}{|}}{\overset{\overset{Cl}{|}}{C}} \cdot COOH$ (dalapon)	76	99
IV	$HOCH_2 \cdot \underset{\underset{CH_3\ Cl}{|}}{\overset{\overset{CH_3}{|}}{C}} \cdot CH^a \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot COOH$	12	83
V	$HOCH_2 \cdot \underset{\underset{CH_3\ OH}{|}}{\overset{\overset{CH_3}{|}}{C}} \cdot CH^a \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot SO_3H$	13	53
VI	$HOCH_2 \cdot \underset{\underset{CH_3\ Cl}{|}}{\overset{\overset{CH_3}{|}}{C}} \cdot CH^a \cdot COOH$	13	53

[a] *dl-*

compounds in which chlorine has replaced hydroxyl groups of the metabolites. Two of these chemicals, the ethyl ester of *dl*–4–chloro–2–hydroxy–3,3–dimethylbutyric acid (I) and the lactone of *dl*–2–chloro–4–hydroxy–3,3–dimethylbutyric acid (II), compare favorably with dalapon (III) as inhibitors of ryegrass root growth. A 50-percent

reduction in growth of ryegrass roots was obtained at 0.001 to 0.002 M for each of these three chemicals. The other two chloro-substituted analogs (IV and VI) have toxic properties as great as those of *dl*–pantoyl taurine (V), a commonly used antimetabolite of pantothenate. Percentage inhibitions of ryegrass coleoptile growth for the six chemicals agreed closely with the corresponding root-inhibition data recorded in Table 4.

Discussion

The *in vitro* studies demonstrated that the chloro-substituted aliphatic acid herbicides inhibited the enzymatic synthesis of pantothenate by competing with pantoate for a site on the enzyme. The dissociation constants obtained for each of the six inhibitors indicated that the affinity of these compounds for the enzyme was about equal to, or greater than, the affinity of the competitive substrate. If this site and mechanism of action are involved, *in vivo,* in the plant growth responses effected by dalapon, then evidence for antagonism of dalapon action should be obtained by supplying either pantoate or pantothenate to the intact organism. Evidence for similar antagonisms by β–alanine might be expected in biological systems deficient in β–alanine, but not in organisms capable of synthesizing sufficient β–alanine to support optimum growth.

The evidence for interactions between dalapon and pantoate or pantothenate but not β–alanine in tiller development in barley was in agreement with the hypothesis that dalapon inhibited the enzymatic synthesis of pantothenate *in vivo* by the same mechanism observed *in vitro*. The inability of exogenous pantothenate to overcome completely the dalapon inhibition of total growth (fresh weight) of either oats or barley suggested that physical or chemical processes other than pantothenate-synthesis were probably involved in the mechanisms of action. Nevertheless, the evidence for a protective effect by pantothenate in the presence of sublethal concentrations of dalapon indicated that the enzyme of pantothenate-synthesis was probably one of the more important metabolic sites involved in the phytotoxic action of dalapon. A similar conclusion was reached in the earlier studies on dalapon inhibition of yeast growth (4).

Evidence for β–alanine protection of growth against the inhibitory action of dalapon has been observed with yeast, an organism incapable of synthesizing sufficient β–alanine to support maximum growth. The evidence for β–alanine–dalapon interactions in ryegrass suggested that a similar protective action by β–alanine may be functional in some higher plants. The enzyme data showing uncompetitive inhibition demonstrated that dalapon and β–alanine act independently at the enzyme level. However, the amount of product synthesized by the dalapon-inhibited enzyme was still dependent upon the degree to which the enzyme was saturated with β–alanine. When the enzyme is saturated with substrate a further increase in β–alanine concentration causes no measurable increase in pantothenate synthesis. This relationship is readily observed *in vitro;* and a similar relationship could be true *in vivo*. Therefore, evidence

for β–alanine antagonism of dalapon inhibitions of growth may be expected with organisms in which β–alanine is a growth-limiting factor. Lack of evidence for a β–alanine-protective effect in some organisms, therefore, does not negate the mechanism of action theory postulated from the *in vitro* studies.

Pantothenate treatments to overcome the phytotoxic effects of dalapon were partially effective in the experiments with barley and oats. Therefore, preliminary experiments (not reported) were initiated with pantothenate-treated seeds of corn, soybean, barley and oats to determine whether the metabolite could reduce dalapon damage to these crop plants. Dalapon was applied to soil containing pantothenate-treated seeds by the macro-screening technique described by Shaw and Swanson (10) for pre-emergence-herbicide-evaluation studies. These preliminary investigations were discontinued when evidence for pantothenate "protection" had been observed to be too inconsistent for the antagonisms to assume practical importance.

When an attempt was made to predict new chemicals which might limit plant growth by interfering with pantothenate metabolism, there came to mind the work on inositol and γ–hexachlorocyclohexane (5, 11). The chemical structure of the antimetabolite is identical with that of inositol except that each of the six hydroxyl groups of the metabolite is replaced with a chlorine atom. This led us to evaluate the phytotoxic properties of several new compounds in which chlorine was substituted for hydroxyl groups in the structure of pantoic acid. The tendency of γ-substituted butyric acids to revert to the lactone hampers the preparation of the analogs as purified acids. Nevertheless, a competitive enzyme inhibitor was obtained from the successful substitution of chlorine for the α–hydroxyl of pantoate. The effect of chloro-substitution on the γ–carbon of pantoate was not studied *in vitro,* but the ethyl ester of the γ–chloro-substituted analog possessed herbicidal activity on ryegrass which was comparable to the activity of dalapon. This analog is of particular interest since the γ–hydroxyl of pantoate is esterified with the pyrophosphate group in the coenzyme A molecule. The phytotoxicity of the four analogs suggests that other pantoate and pantothenate analogs should be evaluated for their herbicidal properties. The recent report that foliar sprays of sublethal concentrations of Na 2,3–dichloroisobutyrate prevent pollen development on cotton (2) suggests that the analogs might also be evaluated for plant "gametocide" activity.

Summary

1. The hypothesis that the enzyme of pantothenate-synthesis is a metabolic site of dalapon action was confirmed *in vitro* and the mechanism of this action defined as competition by dalapon with pantoate for a site on the enzyme of synthesis. Similar results were obtained with several other chloro-substituted aliphatic acids.
2. Evidence for antagonisms between metabolites and dalapon on the growth response of plants treated with sublethal concentrations

of the herbicide is consistent with the mechanism described from *in vitro* studies and implicates the enzyme of pantothenate-synthesis as one of the important sites of dalapon action in plants.

3. Phytotoxicity was predicted for several new pantoate analogs in which the hydroxyl groups of the metabolite were replaced with chlorine. Four such compounds were synthesized and their herbicidal activities on ryegrass seedlings were subsequently demonstrated.

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